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Effect of Simvastatin on Immune Cells under Alkaline and Acidic Conditions

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Abstract

Many clinical investigations have suggested that statins are useful chemotherapeutics against various cancers, whereas *in vitro* experiments using cancer cell lines have shown little effect of statins on cell proliferation and survival. Our group previously demonstrated that statins were preferentially cytotoxic against HeLa, mesothelioma, and pancreatic tumor cells under acidic conditions. A serious side effect of anti-cancer drugs used now is the impairment of the immune system. In this study, we examined the effect of simvastatin on the immune cell lines THP-1 and Jurkat in alkaline and acidic media. Our data suggest that simvastatin inhibited proliferation, survival, and cytokine production at an acidic pH in these cells, whereas the inhibitory effect was negligible at an alkaline pH. These results suggest that anti-cancer drugs whose efficacy increases in acidic cancer nests are useful for potent chemotherapeutics against cancer without causing serious damage to the immune cells in blood and normal tissues, whose pH is slightly alkaline, although the functions of immune cells that have infiltrated acidic cancer nests may be attenuated.

Keywords: Anti-cancer drugs; Statins; Immune cells; Acidic pH; Microenvironment

Introduction

In mammals, blood and normal tissues are maintained within a narrow pH range around 7.4, mainly through the regulation of respiration and renal acid extrusion [1,2]. The extracellular pH, however, decreases to a value below 6 due to the destruction of blood vessels in cancer nests, resulting in the enhancement of hypoxic metabolism that produces lactic acid [3]. It has been reported that cancer nests are acidified by lactic acid accumulation, which is caused by the enhancement of glycolysis combined with impaired mitochondrial oxidative phosphorylation, even if the oxygen supply is not impaired [4].

The pH alteration in cancer nests affects cellular metabolic pathways because all enzyme activities are dependent on the pH. The acidification of cancer nests may affect the functions of target molecules of anti-cancer drugs. Our group previously found that the inhibitory efficacy of statins, manumycin A, and cantharidin increases at an acidic pH [5,6]. Statins are a competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme for the biosynthesis of cholesterol and higher isoprenoids, such as farnesyldiphosphate and geranylgeranyldiphosphate [7]. The isoprenoids are used for protein prenylation. Simvastatin is one of statins and a secondary metabolite produced by fungi [7]. The acidosis-dependent inhibition of cancer cells by statins was caused by the attenuation of protein geranylgeranylation [8], suggesting that a prenylated protein(s) functions to support cell proliferation at an acidic pH. Unfortunately, such a protein(s) remains to be unidentified.

Immune cells infiltrate various cancer nests [9-13] and suppress cancer growth [13]. The acidification of cancer nests may affect immune cell functions, such as cytokine production. The inductions of human dendritic cell maturation [14] and TNF secretion from macrophages were observed under acidic conditions [15]. Extracellular acidosis stimulated IL-1β secretion by human monocytes without affecting the production of TNF-α [16]. Our group demonstrated that extracellular acidic environments enhanced T-cell signaling induced by TCR stimulation, followed by the increase in phosphorylation of TCR signal proteins [17].

Similar acidic environments were also associated with rheumatoid arthritis. The pH of articular fluid in the rheumatoid human joint knee was reported to be around 6.6, compared to around 7.3 in normal joints [18]. Other studies also showed the acidification of synovial fluid in the rheumatoid joints [19-21]. Our group demonstrated that synovial cell survival was preferentially inhibited by statins at an acidic pH [8]. Immune cells are present in the rheumatoid human joint knee [22].

These previous observations revealed the effectiveness of acidosis-dependent drugs for chemotherapeutics against cancer cell growth and inflammation. It remains unclear how such drugs affect immune cell functions in acidic, diseased areas. In the present study, we found that simvastatin and manumycin A preferentially suppressed the proliferation and survival of immune cell lines at an acidic pH, and that the statin attenuated the production of TNF-α, IL-1β, and IL-8 under acidic conditions.

Materials and Methods

Cells and reagents

Human monocytic cell lines THP-1 and Jurkat E6.1 derived from human T cells were donated by Itsuko Ishii (Chiba University, Japan) and Takashi Saito (RIKEN, Japan), respectively, and cells were cultured at 37°C under 5% CO_2 in RPMI-1640 containing 24 mM $NaHCO_3$, 10 μg/mL gentamycin, 5 μg/mL fungizone, and 10% fetal bovine serum (FBS).

Simvastatin was purchased from Wako (Osaka, Japan). In the

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indicated experiments, the statin was converted to the open ring form before use, as described previously [23]. Manumycin A (Wako) and YM-53601 (Sigma-Aldrich) were purchased. Cell Counting Kit 8 was purchased from Dojindo (Kumamoto, Japan).

Media for cell proliferation and survival assays under different pH conditions

Media at various pH values for cell proliferation and survival assays were prepared as follows. To minimize the pH change during cell culture, 10 mM PIPES [piperazine-*N*,*N*'-bis (2-ethanesulfonic acid)] for acidic media or HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] for alkaline media was added to RPMI-1640 instead of $NaHCO_3$. Medium containing FBS was often contaminated with microbes when the medium pH was adjusted. Therefore, the medium pH was first adjusted by the addition of NaOH to medium without FBS. After sterilization of the medium by filtration, FBS was added. Since the medium pH was changed by the addition of FBS, the pH of medium without FBS was adjusted to a lower and higher value than the final pH in acidic and alkaline media, respectively. For example, when media of pH 6.1, 6.7, and 7.5 containing 10% FBS were used, the pH values were adjusted to 5.8, 6.4, and 7.6, respectively, before the addition of FBS. An inhibitor was added after the addition of FBS when indicated.

Inhibitory effect of various inhibitors on cell survival at different pH values

Cells were suspended in pH 7.5 RPMI-1640 medium prepared as described above, and 50 μL of the cell suspensions was placed in 96-well plates at 7.5×10^2 cells/well. After incubation for 1 day at 37°C without a CO_2 supply, 100 μL of pH 6.1 RPMI-1640 medium containing the indicated inhibitor was added to the wells. The pH of the resulting mixture was 6.7. For incubation at pH 7.5, 100 μL of pH 7.5 RPMI-1640 medium containing the indicated inhibitor was added to the wells. Culture plates were put in the box with sterilized water, and then the box was covered with a loose lid to minimize evaporation of the medium. The box was incubated at 37°C in an incubator without a CO_2 supply. Cells were cultured for 5 days without a CO_2 supply, and cell survival was determined with Cell Counting Kit 8.

Inhibitory effect of various inhibitors on cell proliferation at acidic pH

Cells were suspended in RPMI-1640 media of pH 6.7 or 7.5 prepared as described above at 1.4×10^5 cells per mL, and inhibitors indicated were added. Cells were cultured for 4 days without a CO_2 supply, and cell proliferation was determined by counting the number of cells not stained with trypan blue before and after incubation.

Real-time RT-PCR

Total RNA was isolated using TRI reagent (sigma-Aldrich) from cells incubated for 2 days at different pH values with or without simvastatin, and 1 μg of total RNA was subjected to RT reaction. The cDNAs were subjected to real-time RT-PCR using Fast Start Universal SYBR Green Master ROX (Roche) with the ABI7000 system (Applied Biosystems). 18S rRNA was used as a control RNA. Since the amount of ribosomes in mammalian cells is 4×10^6 per cell [24], the amount of mRNA relative to that of 18S rRNA gives an approximate copy number of mRNA per cell. The primers used are described in Table 1. Sequences of TNF-α, IL-1β, IL-8, and 18S rRNA were quoted from NM_000594.2, NM_000576.2, NM_000584.2, and [25], respectively.

Statistical analysis

The Student's t-test was utilized in this study.

Inhibition of cell proliferation and survival of THP-1 cells by various inhibitors. (A) The p values compared with data obtained without simvastatin at both pH values were calculated. *p<0.01; no mark, p<0.01. (B to D) the p values at pH 6.7 compared with data at pH 7.5 were calculated. *p<0.01; no mark, p>0.01.

Inhibition of cell survival of Jurkat cells by simvastatin, manumycin A, and YM-53601. The mean values and S.D. obtained from three experiments using different cultures are represented. S.D. less than 10% is not represented. The p values at pH 6.7 compared with data at pH 7.5 were calculated. *p<0.01; no mark, p>0.01.

Expression of cytokine genes in THP-1. The mean values and S.D. obtained from three experiments using different cultures are represented. The p values compared with data obtained without simvastatin at both pH values were calculated. *p<0.01; no mark, p>0.01. The p values at pH 6.7 compared with data at pH 7.5 were calculated in the absence of simvastatin. **p<0.01.

Results

Inhibition of proliferation and survival at acidic pH

When cells were cultured under our conditions, the medium pH decreased and increased continuously in alkaline and acidic media, respectively. After 5 days culture, the medium pH values were 7.3 and 6.8 when media of pH 7.5 and 6.7 were used, respectively.

Simvastatin at 2 μM suppressed the proliferation of human monocyte THP-1 cells only at pH 6.7 (Figure 1A). The effect of simvastatin on cell survival was subsequently examined, and the results showed that 5 μM simvastatin markedly reduced THP-1 survival at pH 6.7, while it had no significant effect on survival at pH 7.5 (Figure 1B). Statins exist in two forms, a lactone form and open ring hydroxy acid form [26]. It was reported that the lactone form is absorbed from the gastrointestinal tract and transformed into the open ring form *in vivo* [27]. Similar results were obtained with the two forms. The survival decreased with a lower concentration of manumycin A at pH 6.7 than that at pH 7.5 (Figure 1C), whereas the difference in survival between the two pH conditions was small in cells treated with YM-53601 (Figure 1D). Statins are inhibitors of HMG-CoA reductase (Figure 1E). Manumycin A and YM-53601 inhibit protein prenylation and cholesterol synthesis, respectively (Figure 1E). These results were similar to those obtained with mesothelioma [5] and synovial cells [8]. Similar results were also obtained in Jurkat T cells derived from human T cells (Figure 2). Protein prenylation has been suggested to be a pathway required for the proliferation and survival of any type of cell under acidic conditions.

Expression of cytokines under acidic conditions

The effect of simvastatin on the expression of cytokine genes was subsequently examined in THP-1 cells. Levels of TNF-α and IL-1β produced by immune cells were suggested to have inhibitory effects

Gene		Sequence	Size
TNF-α	forward	CCCCAGGGACCTCTCTCTAATC	98
	reverse	GGTTTGCTACAACATGGGCTACA	
IL-1β	forward	GGACAAGCTGAGGAAGATGC	120
	reverse	TCGTTATCCCATGTGTCGAA	
IL-8	forward	TCTGGCAACCCTAGTCTGCT	136
	reverse	GCTTCCACATGTCCTCACAA	
18S rRNA	forward	TAGAGTGTTCAAAGCAGGCCC	81
	reverse	CCAACAAATAGAACCGCGGT	

Table 1: Primers used in this study.

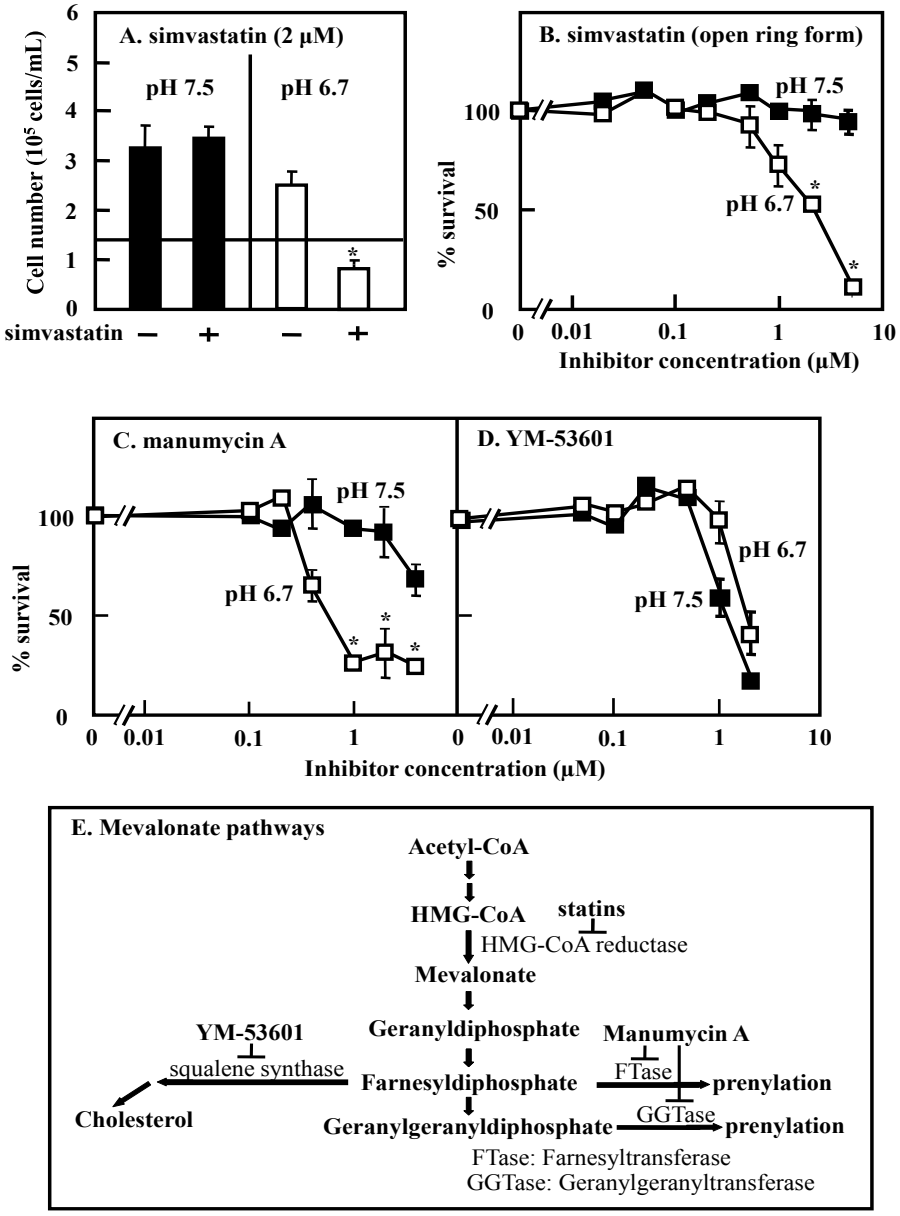

Figure 1: Inhibition of cell proliferation and survival of THP-1 cells by various inhibitors. (A) THP-1 cells were cultured in RPMI-1640 media at pH 6.7 or pH 7.5 for 4 days with simvastatin at the concentrations indicated, and cell numbers were measured as described in Materials and Methods. The dotted line represents the cell number before culture. The mean values and S.D. obtained from two experiments using different cultures are represented. The p values compared with data obtained without simvastatin at both pH values were calculated. *p<0.01; no mark, p>0.01. (B) to (D) THP-1 cells were cultured in RPMI-1640 media at pH 6.7 or pH 7.5 for 5 days with the inhibitors indicated, and cytotoxicity was measured as described in Materials and Methods. Absorbance obtained without an inhibitor was taken as 100%. The mean values and S.D. obtained from three experiments using different cultures are represented. S.D. less than 10% is not represented. The p values at pH 6.7 compared with data at pH 7.5 were calculated. *p<0.01; no mark, p>0.01. (E) Mevalonate pathways.

on tumor progression [28,29]. TNF-α was up-regulated 10-fold under acidic conditions compared with that at pH 7.5 in THP-1 cells, and the increased gene expression was significantly suppressed by simvastatin at pH 6.7 (Figures 3A and B). The expression of IL-1β was enhanced at an acidic pH, and this elevated expression was reduced by simvastatin in THP-1 cells (Figures 3C and D). Similar effects of the extracellular pH and simvastatin on the expression of IL-8 were observed (Figures 3E and F). These results suggest that expression of the inflammatory cytokines TNF-α, IL-1β, and IL-8 was enhanced in cancer nests associated with extracellular acidosis, and that the expression was inhibited by statins. The expression of IL-8 at an acidic pH was reported in tumor cells [30,31]. Since the mRNA levels of IL-1β and IL-8 were very low compared with that of TNF-α (Figures 3B, D, and F), the expressions of IL-1β and IL-8 may be less significant physiologically.

Simvastatin increased the expression of TNF-α, IL-1β, and IL-8 at 5, 2, and 2 μM, respectively, in alkaline medium (Figure 3), while this drug inhibited the expression in a dose-dependent manner at an acidic pH. It remains unclear why simvastatin increases the expression of these cytokines.

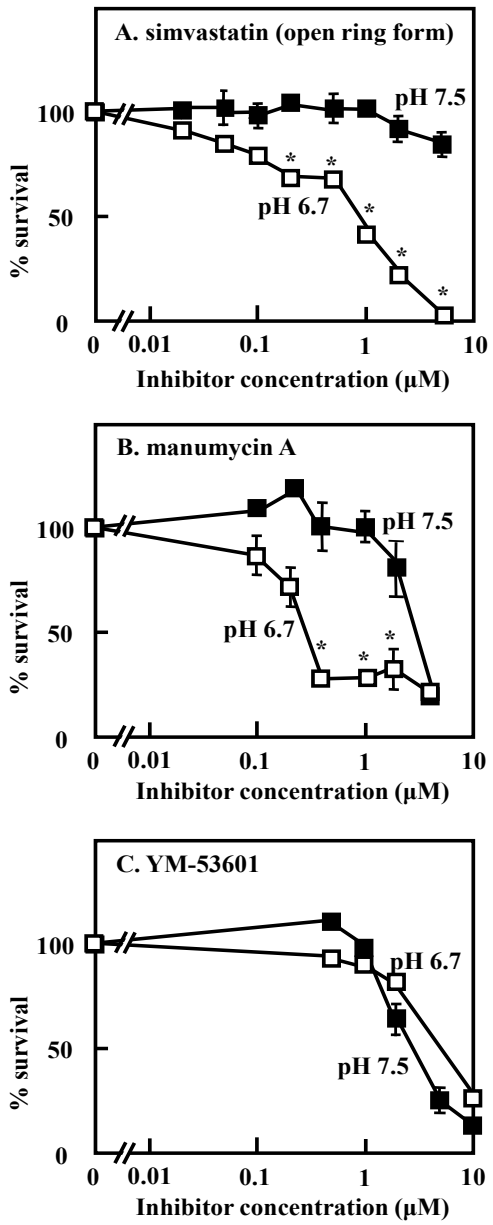

Figure 2: Inhibition of cell survival of Jurkat cells by simvastatin, manumycin A, and YM-53601. Jurkat cells were cultured in RPMI-1640 media at pH 7.5 or 6.7 for 5 days with simvastatin, manumycin A, or YM-53601 at the concentrations indicated, and cell survival was measured as described in the legend of Figure 1B. The mean values and S.D. obtained from three experiments using different cultures are represented. S.D. less than 10% is not represented. The p values at pH 6.7 compared with data at pH 7.5 were calculated. *p<0.01; no mark, p>0.01.

Discussion

Statins were reported to inhibit the proliferation and survival of cancer [5] and synovial [8] cells under acidic conditions. The acidosis-dependent inhibition by simvastatin was observed in immune cells in the present study. Cancer nests and inflammatory loci were acidified by the limitation of oxygen [3,18-21]. It is therefore suggested that statins have a marked effect on any type of cell under acidic conditions which are close to those in cancer nests. When the box was sealed, cells were damaged after 4 to 5 days of culture, suggesting that oxygen is required for proliferation at an acidic pH. It was essential for preventing the cell damage to take off the lid of the box for a while every two days. The oxygen level might decrease during culture in the box.

The suppressive effect of statins on T cells has already been reported. Atorvastatin inhibits T-cell activation and proliferation [32]. In their report, the inhibition of Jurkat cell proliferation by 10 μM atorvastatin was approximately 60%, being close to our results obtained with simvastatin at pH 7.5 (Figure 2A). The association of signaling proteins including Lck to membrane rafts was reported to be inhibited by statins with decreasing cholesterol synthesis [32]. In addition to the effect on lipid rafts, the inhibition of protein geranylgeranylation by statins was found to be critical for T-cell functioning [33]. The prenylation of small G proteins was inhibited by statins, and the inhibition affected several intracellular functions including cell proliferation [34]. These data may support the mechanism in which the effect of statins on cell proliferation and survival is due to the attenuation of a geranylgeranylated protein(s). This mechanism would be more important under acidic conditions because sensitivity to statins was enhanced at a low pH and manumycin A preferentially suppressed cell survival at a low pH (Figure 1). G protein-coupled receptor on T-cell membranes encoded by T-cell death-associated gene 8 (TDAG8) was assumed to be a pH sensor to regulate intracellular cAMP under acidic environments [35]. Ras was reported to be more active at a low pH and to activate cAMP-dependent kinase (PKA) [36], suggesting that PKA signaling has an important role in cellular survival at an acidic pH.

Our present results demonstrated that TNF-α was expressed at a higher level at an acidic pH compared with that at an alkaline pH in THP-1. Simvastatin prevented TNF-α-induced NF-κB activation, which ultimately results in the secretion of pro-inflammatory cytokines [37]. Our results showed the significant inhibition of TNF-α expression by simvastatin at an acidic pH. Simvastatin also significantly inhibited the production of IL-1β and IL-8 in THP-1 (Figure 3). Some statins were reported to induce apoptosis via caspase-3 activation [38], but such activation was not observed in the present study (data not shown).

The protein synthesis may be dependent on a copy number of mRNA, because the binding chance of mRNA to ribosomes declines as the decrease in the copy number. The copy number of mRNA was calculated based on the content of ribosomes per cell in this study. The content is written in the textbook [24], and this number was used. The content was also calculated based on the published data [39], and the same result was obtained. No data concerning the content of ribosomes in cells cultured at an acidic pH has been reported. The content of 18S RNA was measured using PCR in this study, and Ct values were almost the same in all cells cultured in this study (data not shown). We, therefore, assumed that the content of ribosomal RNA is constant even if the culture pH is different.

TCR stimulation did not increase cytokine expression at an acidic pH in Jurkat cells [40] or human peripheral primary T cells (unpublished observation). The mRNA levels of interleukins and TNF-α were less than 100 copies per cell except IL-10, while the mRNA level of GAPDH was 8×10^5 copies per cell in Jurkat cells [40]. Thus, the expression of these cytokines might be less significant, and the effect of simvastatin on the expression of cytokines was not examined in Jurkat cells in this study.

Our present data showing that cytokine production was inhibited by statin treatment under acidic conditions do not seem to be of merit for cancer therapy. In contrast to an acidic pH, treatment with acidosis-dependent drugs was less effective on immune cells at an

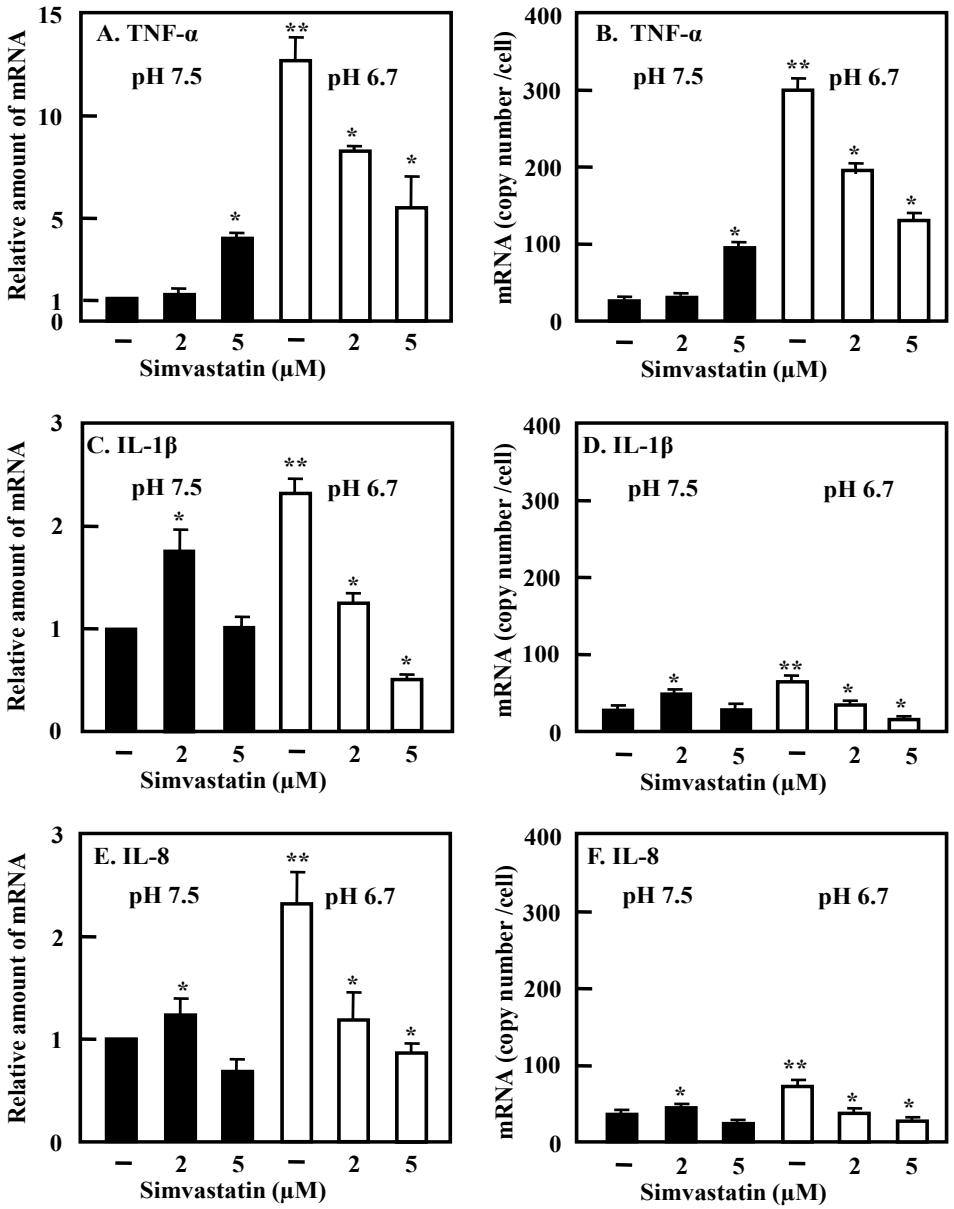

Figure 3: Expression of cytokine genes in THP-1. After THP-1 cells had been cultured in RPMI-1640 media at pH 7.5 or 6.7 for 2 days with simvastatin at the concentrations indicated, the amounts of indicated mRNA were measured as described in Materials and Methods. The left column: Relative increases in mRNA levels of TNF-α (A), IL-1β (C), and IL-8 (E) are represented. The right column: Copy numbers of mRNA per cell of TNF-α (B), IL-1β (D), and IL-8 (F) are represented. The mean values and S.D. obtained from three experiments using different cultures are represented. The p values compared with data obtained without simvastatin at both pH values were calculated. *p<0.01; no mark, p>0.01. The p values at pH 6.7 compared with data at pH 7.5 were calculated in the absence of simvastatin. **p<0.01.

alkaline pH that is close to the pH of normal tissues including blood, suggesting that immune systems could be maintained more actively in the body when acidosis-dependent anti-cancer drugs are used. Statins are now prescribed as drugs to decrease the blood level of cholesterol, and damage of the immune system has not be reported to date in hyperlipidemia patients treated with them. Stains were reported to increase incident diabetes, but the adjusted hazard ratio was 1.04 to 1.17 [41]. The risk of a major cardiovascular event was less than 1 % in patients treated with statins [42]. Chemotherapy combined with immunotherapy is now being developed. It may become possible to create immune cells in which cytokine production is resistant to statins.

It was shown that the expression of many genes was affected by environmental pH [43]. Some acidosis-dependent genes were shown to be expressed at a higher level in specimens from cancer patients [44]. The present study demonstrated that the efficacy of some medicines increases under acidic conditions. These data suggest the significance of measuring pH levels inside human body and the pH change of diseased areas for our improved understanding of therapy and diagnosis.

In conclusion, simvastatin was shown to be less effective at a slightly alkaline pH, being close to that of blood and normal tissues, although this drug exhibits cytotoxicity against immune cells in an acidic medium. Acidosis-dependent drugs such as statins can be

argued to have weak side effects on the immune system, as shown in the present study. Impairment of the immune system is one of the serious side effects of anti-cancer drugs used commonly now. Our results lead us to anticipate that the screening of chemicals exhibiting high-level cytotoxicity in acidic medium will promote the development of new medicines for chemotherapeutics against cancer with reduced side effects.

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